Health Protection Agency

DECLARATION

I, the undersigned, Dr Keith Foster, of Health Protection Agency, do solemnly and sincerely declare that I am an inventor of US 5,989,545 and US 6,395,513, which relate to clostridial protease conjugates and to the preparation thereof.

As evidenced by the abridged version of my *curriculum vitae* (Annex 1), I have been actively undertaking research in the technical field of protein chemistry for the last 27 years. I am therefore entirely familiar with protein conjugation chemistry.

I also confirm that I am familiar with the prosecution history (to date) of US 09/529,130.

The methodology involved in coupling two protein molecules (A and B) together is simple, and is achieved through the use of a cross-linking agent (also known as a chemical coupling agent). For example, molecules A and B are separately contacted with a cross-linking agent, which chemically modifies a specific surface group on each of molecules A and B thereby forming derivatised molecules A' and B'. The modified surface group on molecule A' is capable of covalently bonding with the modified surface group on molecule B'. Thus, the coupling reaction is completed by mixing together the two protein molecules A' and B'.

Chemical coupling agents have been commercially available for many years, in particular, well before the priority date (8 October 1997) of the present application. Moreover, the use of cross-linking agents has been widespread for many years (certainly prior to 8 October 1997).

Example 1 of US 09/529,130 illustrates the use of one such coupling agent, namely SPDP, to chemically couple two protein molecules (a galactose-

binding lectin, and the LH_N of botulinum neurotoxin). The two molecules are separately contacted with SPDP, and then mixed together to allow covalent conjugation.

In addition, lines 5-7 on page 8 of WO99/17806 (from which US 09/529,130 is derived) confirm that the claimed agent can be produced according to WO96/33273 - an earlier patent application for which I am a named inventor. This confirmation is repeated at lines 16-18 on page 9 of WO99/17806 which explicitly states that "[t]hese and other lectins of the sub-class of galactose-binding lectins can be used as targeting moieties (TM) for conjugates of the type described in WO96/33273."

The conjugates described in the Examples of WO96/33273 confirm that PDPH/EDAC, or Traut's reagent may be employed as an alternative chemical coupling agent to SPDP. Moreover, referring to the specification of WO96/33273 (see the paragraph that follows the Examples and immediately precedes Table 1), it is clear that:-

> *"**any other** coupling chemistry capable of covalently attaching the TM component of the agent to [the] clostridial neurotoxin derived component and known to those of skill in the art is covered by the scope of this application"* [emphasis added].

WO99/17806 (from which US 09/529,130 is derived) also describes at page 9, lines 28-31, using any coupling chemistry capable of attaching the lectin component to the LH_N component of the claimed agents: "Thus, in one embodiment of the invention a galactose-binding lectin is conjugated, using linkages that may include one or more spacer regions, to a derivative of the costridial neurotoxins." WO99/17806 also states at page 10, lines 15-19 that the "TM, L or LH_N and translocation domain components may be separately

expressed in a recombinant form and subsequently linked, covalently or non-covalently, to provide the desired agent."

Hence, the skilled artisan would recognize that US 09/529,130 generically describes covalently linking certain lectins to LH_N. Moreover, as further described below, the skilled artisan at the time of filing US 09/529,130 was familiar with chemical coupling agents and their use for covalently linking proteins. Thus, the skilled artisan would also have recognized that the generic description of covalent linkages that may include one or more spacer regions is a well known class of compounds useful for chemically coupling proteins.

SPDP was illustrated as the cross-linking agent in Example 1 of US 09/529,130 simply because it was (and still is) a popular and well-documented coupling agent in the technical field of protein conjugation chemistry. Thus, SPDP is simply one example of a well known class of compounds that may be employed to covalently link together the Targeting Moiety component and the clostridial neurotoxin component of the conjugate described in US 09/529,130. For example, I have managed research groups who have employed SMPB, SMCC (succinimidyl 4-(*N*-maleimidomethyl) cyclohexan-1-carboxylate), and Traut's reagent as alternative chemical coupling agents to link together a galactose-binding lectin or an N-acetylgalactosamine-binding lectin and LH_N.

Whilst the specification of US 09/529,130 does not provide an exhaustive list of chemical coupling agents, I do not consider this places any undue burden on a person of skill in the art seeking to reproduce the present invention across the scope of the present claims (ie. Claims 63, 65, 66 and 71 as filed in response to the Official Action dated 9 July 2004). This is because the use of coupling agents for joining together two components of the defined agent is (and was prior to October 1997) a matter of routine to a person of skill in the art.

In more detail, commercially available members of the well known class of coupling agents may be used for conjugation purposes to produce an agent of the invention described in US09/529,130. By way of example, I refer to the following pre-1997 publications:-

Annex 2 - Hermanson, G.T. (1996), Bioconjugate techniques, Academic Press;

Annex 3 - Wong, S.S. (1991), Chemistry of protein conjugation and cross-linking, CRC Press;

Annex 4 - Thorpe *et al* (1987), Cancer Res, 1987, 47, 5924-31. This paper describes the use of SMBT (sodium S-4-succinimidyloxycarbonyl-alpha-methyl benzyl thiosulfate) and SMPT (4-succinimidyloxycarbonyl-alpha-methyl-alpha(2-pyridyldithio)toluene); and

Annex 5 - Peeters *et al* (1989), J Immunol Methods. 1989, 120, 133-43. This paper describe the use of 4 coupling reagents, MHS (succinimidyl 6-(N-maleimido)-n-hexanoate), SMCC (succinimidyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate), MBS (succinimidyl m-maleimidobenzoate), and SPDP.

Whilst it is true that the use of different coupling agents may have different effects (ie. improved, neutral, or adverse) on the biological activity of an agent according to US 09/529,130, I can confirm that the use of such coupling agents is (and was prior to October 1997) a matter of routine to a person of skill in the art. Thus, in principle, any member of the well known class of chemical coupling agent may be employed to couple a lectin to a polypeptide comprising a clostridial protease domain and a clostridial translocation

domain. Moreover, having selected a particular coupling agent, it is (and was prior to October 1997) a matter of routine to confirm that the resulting agent has the requisite biological activity, as explained in more detail below.

Confirmation of L-chain function after chemical coupling may be tested by assaying for protease activity inherent to the L-chain.

By way of example, any one of the following three routine tests may be employed.

SNAP-25 (or synaptobrevin, or syntaxin) may be challenged with an "agent" to be tested, and then analysed by SDS-PAGE peptide separation techniques. Subsequent detection of peptides (eg. by silver staining) having molecular weights corresponding to the cleaved products of SNAP-25 (or other component of the neurosecretory machinery) would confirm the presence of a functional L-chain.

As an alternative, the "agent" may be tested by either *in vitro* challenge (see Examples 4-7 of US 09/529,130) or *in vivo* challenge in a mouse experiment (see Examples 8-9 of US 09/529,130).

As a further alternative, the "agent" may be tested by assaying for SNAP-25 (or synaptobrevin, or syntaxin) cleavage products via antibody-specific binding (see WO95/33850). In more detail, a specific antibody is employed for detecting cleavage of SNAP-25. Since the antibody recognises cleaved SNAP-25, but not uncleaved SNAP-25, identification of the cleaved product by the antibody confirms the presence of L-chain proteolytic function. By way of exemplification, such a method is described in Examples 2 and 3 of WO96/33273.

Confirmation of H-chain function after chemical coupling may be tested by assaying for translocation activity inherent to the H-chain.

Suitable methods are, for example, described by Shone *et al.* (1987) Eur. J. Biochem. 167, pp.175-180, and by Blaustein *et al.* (1987) FEBS 226 (1), pp.115-120. Copies of these documents are provided as Annex 6 and Annex 7.

The Shone *et al.* method employs artificial liposomes loaded with potassium phosphate buffer (pH 7.2) and radiolabelled NAD. Release of K^+ and NAD from the liposomes correlates with a positive result for channel forming activity and hence translocation activity. In this regard, K^+ release from liposomes may be measured using an electrode and NAD release calculated by measuring the radioactivity in the supernatant (see page 176, column 1, line 33 - column 2, line 17).

The Blaustein *et al.* method employs planar phospholipid bilayer membranes, which are used to test for channel forming activity. In more detail, salt solutions on either side of the membrane are buffered at a different pH - on the *cis* side, pH 4.7 or 5.5 and on the *trans* side, pH 7.4. The "agent" to be tested is added to the *cis* side of the membrane and electrical measurements are made under voltage clamp conditions, in order to monitor the flow of current across the membrane (see paragraph 2.2, pages 116-118). The presence of an active translocation function is confirmed by a steady rate of channel turn-on (i.e. a positive result for channel formation) -see paragraph 3, page 118.

Confirmation of Targeting Moiety (TM) function after chemical coupling may be tested by assaying for galactose-binding function inherent to the TM.

Suitable methods include:-

a simple haemagglutination assay [see, for example, Iglesias, J.L. *et al* (1982) – Purification and properties of a D-galactose/ N-acetyl-D-galactosamine-specific lectin from *Erythrina cristagalli*. Eur. J. Biochem., 123, pp. 247-252 – see Annex 8; and Arango, R. et al (1992) – Expression of *Erythrina corallodendron* lectin in *Escherichia coli*. Eur. J. Biochem., Apr 15, 205, pp. 575-581 – see Annex 9]; or

a simple assay to test for the ability to bind to immobilised sugars (eg. galactose) - see Iglesias, J.L. *et al* (1982) – Purification and properties of a D-galactose/ N-acetyl-D-galactosamine-specific lectin from *Erythrina cristagalli*. Eur. J. Biochem., 123, pp. 247-252

Haemagglutinin assays are well known in the art. In this regard, erythrocytes are known (and were so prior to 8 October 1997) to possess surface sugar (eg. galactose) residues, and are therefore susceptible to agglutination by galactose-binding lectins. Thus, the agglutinating activity of a galactose-binding lectin may be confirmed using heparinised human group O, Rh0 (D)+ erythrocytes. In more detail, the erythrocytes are prepared by trypsinisation, and 90 min incubation at room temperature. The resulting suspension is then challenged with an "agent" to be tested (diluted in PBS) and agglutination activity screened visually thereafter.

Immobilised sugar assays are well known in the art. In this regard, it is (and would have been prior to 8 October 1997) routine to test for active galactose-binding function. In more detail, a defined quantity of sugar [eg. N-acetyl galactosamine (GalNAc), or immobilised galactose (Gal)] is immobilised on to a matrix (eg. agarose). A "control" galactose-binding lectin mixture is added to the matrix and tumbled gently at room temperature (eg. for 30 minutes). The mixture is then transferred from the tumbling vessel to an empty 2.5ml

polypropylene column and the eluant collected. After several column volume washes [eg. with 20 mM Hepes buffer pH 7.0], the buffer is switched to include 0.3 M GalNAc or Gal, with the result that specifically bound material is eluted. Using standard protein quantitation methodology (eg. BCA assay, Bradford assay, absorbance at 280nm), the ratio of unbound to specifically eluted material is readily estimated. This ratio may be used to determine the effect of coupling chemistry on the binding activity of the lectins.

Summary

I firmly believe that the specification of US 09/529,130 provides adequate written description and enablement details for a person of skill in the art to reproduce the invention across the scope of the pending claims (ie. Claims 63, 65, 66 and 71 as filed in response to the Official Action dated 9 July 2004).

I make this solemn declaration conscientiously believing the same to be true;

Signed .....

Witnessed M Rhodes .....

Dated 15 Nov. '04 .....